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13. ABSTRACT *(Maximum 200 Words)*

This is an IDEA Award in which we propose to characterize the molecular events driving acquired antiestrogen resistance. We have begun to acquire extensive gene expression microarray data on a panel of eight different antiestrogen resistant cell lines and their parental cells. The analysis of these data is ongoing and the study is on-track. Several new algorithms for data analysis are underdevelopment. One of these algorithms, a new method for data normalization, has been developed and tested and a manuscript is now in press. Our data with NFκB and IRF-1 suggest we may be on the right track to identifying new signal transduction pathways associated with acquired antiestrogen resistance. For example, these data show that resistant cells are more sensitive to inhibition of NFκB. Overexpression of IRF-1, which is suppressed by estrogens and induced by antiestrogens, is associated with reduced cell proliferation.

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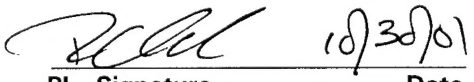

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Introduction

Antiestrogens have been successfully used in the management of breast cancer since the first clinical trial of Tamoxifen (TAM) in 1971 (1). TAM produces a significant increase in both overall and recurrence-free survival but resistance almost inevitably arises in most patients (2,3). **We hypothesize that one form of acquired antiestrogen resistance reflects the altered expression of what were previously estrogen regulated genes. We further hypothesize that only a subset of all estrogen (E2)-regulated genes, those comprising a specific gene network, is responsible for the resistance phenotype.** Since TAM (triphenylethylene) and ICI 182,780 (steroidal) induce different ER conformations, **we also hypothesize that the consequent patterns of gene regulation will be different and dictate the presence/absence of crossresistance among antiestrogens.**

To address these hypotheses, we have generated novel E2-independent and antiestrogen resistant variants of the E2-dependent, MCF-7 human breast cancer cell line (MCF7/MIII, MCF7/LCC1, MCF7/LCC2, MCF-7/LCC9) - recently reviewed in (4). We also have assembled a panel of additional resistant cells from within this institution and from other investigators. These include additional antiestrogen resistant MCF-7 variants (LY2, R27, R3, MCF-7RR), all of which express ER, and the ER-negative ZR-75-1 (ZR75/LCC3, ZR-75-9a1) and T47D (T47Dco) variants. Other resistance models are currently being obtained from other laboratories or being generated by selection *in vivo* selection against TAM in athymic nude rats (rats and humans perceive TAM as a partial agonist, mice perceive TAM as a pure agonist).

This is an Idea Award to study the genes and patterns of genes expressed in acquired antiestrogen resistance in cell culture models. The PI will apply new, state-of-the-art technologies to identify key endocrine-regulated molecular pathways to apoptosis/proliferation. By identifying key components of these pathways, we may be able to predict response to first-line and crossover antiestrogenic therapies, and/or provide novel therapeutic strategies for antiestrogen resistant tumors.

Body of Text

Our purpose is to evaluate a series of antiestrogen responsive and resistant breast cancer cell lines for their patterns of gene expression. We will explore these data, using state-of-the-art clustering pattern analysis through joint use of the standard Finite Normal Mixture models and probabilistic component subspaces, where the multimodal clusters will be automatically identified using Akaike information criterion and Minimal Description Length analyses. We also will apply the more computationally simplistic methods used by others in the field.

We have made one change to the specific aims and Statement of Work. Our collaborations with Dr. Wang's group at Catholic University of America have increased substantially, and we have begun to develop and test several new algorithms for mining the high dimensional data sets produced by gene expression microarray analyses. Thus, we have included some of this work in the Aims and SOW (see bolded text below).

Specific Aims

Specific Aim 1: use gene microarrays to identify differentially expressed genes in a panel of breast cancer cell lines.

Specific Aim 2: explore the data from Aim 1 to identify those differentially expressed gene clusters most closely associated with acquired antiestrogen resistance **and test further novel algorithms for the analysis of gene expression microarray data** (modification in bolded text).

Specific Aim 3: begin to assess the likely functional relevance of representative members of these clusters and study their expression in human breast cancer biopsies.

Long term aims: establish a pattern(s) of gene clusters that can predict antiestrogen responses in patients. This could lead to a more effective identification of candidates for specific antiestrogen therapies and identify those patients least likely to respond and who may benefit from an early initiation of cytotoxic chemotherapy.

Statement of Work and Progress on the Work Proposed

The Specific Aims of this application are being addressed in the studies outlined in the following modified Statement of Work (modification in bolded text).

TASK 1: *Use gene microarrays to identify differentially expressed genes in a panel of breast cancer cell lines.*

A. Expand cells and prepare RNA from cell lines for pilot study
B. Label RNA populations, probe microarrays and digitize data
C. Optimize probing/reprobing as necessary
D. Expand cells and prepare RNA from replicate cultures of remaining cell lines (including ER-negative cells) for the baseline study
E. Label RNA populations, probe microarrays and digitize data
F. Expand cells, treat with ICI 182,780 and 4-hydroxyTAM and prepare RNA from replicate cultures
E. Label RNA populations, probe microarrays and digitize data

We proposed to complete analyses based on 8 resistant cell lines and their respective parental cell lines (both untreated and antiestrogen treated). We began by extracting RNA from three independent cell cultures each of 13 (parental + resistant) of the untreated cells (39 RNA populations total). Of these, we have extracted 31 RNAs. Probes have been made and already hybridized to filters for 18 of these RNA populations. Our initial studies have used the Research Genetics nylon filters, in which we can assess the expression of 4,032 known genes, 192 "housekeeping" genes, and 192 control

genes. We now have full access to the Affymetrix technology and can array samples using both technologies, resources permitting.

The remaining RNA populations from untreated cells should be collected within the next few weeks. We expect to complete arraying and analyzing the data from all the untreated cell populations within the next few months. We will then begin collecting RNA from the cell populations treated with the appropriate antiestrogens and arraying these RNAs.

We used a new method for labeling probes for Research Genetics, Inc. filters that involves using two radionucleotides to label both the sense and antisense probe strands (5). To synthesize the labeled cDNA probe, total (not polyA+) RNA is incubated at 70oC for 10 min with 2 μg of oligo dT and then chilled on ice for 2 min. The primed DNA is incubated at 37oC for 90 min in a solution containing 1x first strand, 3 mM DTT, 1 mM dGTP/dTTP, 300 Units of reverse transcriptase, 50 mCi of [^{33}P] dCTP and 50 mCi of [^{33}P] dATP. The second strand is synthesized by adding 1x reaction buffer, 100 Units DNA polymerase I, 500 ng of random primers, 1 mM dGTP/dTTP, 50 mCi of [^{33}P] dCTP and 50 mCi of [^{33}P] dATP. The reaction is incubated for 2 hrs at 16oC. Labeled probe is purified using a BioSpin-6 chromatography column (Bio-Rad) and denatured by boiling for 3 min. Purified probe is added to the hybridization roller tube containing the prehybridized GeneFilter and is incubated for 12-18 hrs at 42oC in a Robin Scientific Roller Oven. The Hybridized GeneFilter is washed twice in 2x SSC, 1% SDS at 50oC for 20 min and once at 55oC in 0.5x SSC, 1% SDS for 15 min. Probes were hybridized to filters, and signals obtained using a Molecular Dynamics Storm phosphorimager. the analysis if these data is ongoing (see Task 2).

We have also completed the first series of studies described as preliminary data in the original application (data on interferon regulatory factor-1, human X-box binding protein, and nuclear factor kappa B). The data were confirmed/repeated and the functional studies extended (see under Task 3); the studies completed also address issues described under Tasks 2and 3. A manuscript has been written and submitted for publication. A copy of a final accepted manuscript or reprint will be included in our next annual report. We also have published a major review on antiestrogen resistance (4).

TASK 2: *Explore the data from Aim 1 to identify those differentially expressed gene clusters most closely associated with acquired antiestrogen resistance.*

A. Perform preliminary analysis of pilot study and identify candidates for further study
B. Generate reagents and confirm differential regulation/expression of candidates from the pilot study
C. Analyze the data from the baseline study (includes evaluation of ER-negative models both separately and together with ER-positive cell) using all four data analysis approaches and identify candidates for further study
D. Generate reagents and confirm differential regulation/expression of candidates from the baseline study

E. Analyze the data from the treatment study using all four approaches and identify candidates for further study

F. Perform overall and final analyses, compare data from each analytical method and identify candidates for further study

G. Generate reagents and confirm differential regulation/expression of candidates from the treatment study

H. **Test novel algorithms for the analysis of gene expression microarray data**

Data from the first microarray hybridizations are currently being processed and analyzed. Our intent is to use these data to train neural network predictors of responsiveness and resistance to antiestrogens. We have recently been successful in taking this approach to predict the nature of breast core needle biopsies from breast cancer patents. We obtained gene expression microarray data from ten such biopsies, five containing >80% breast cancer and five containing >70% noncancer tissues. Data were obtained using the same microarrays used for the cell culture studies. Dimensionality was reduced to the top discriminatory genes using an approach similar to that described by Hedenfalk *et al.* (6). The goal was to demonstrate our ability to select genes and use these to generate accurate predictive neural network models.

We tested three network configurations (multilayer perceptron-based neural networks), with either 1, 2 or 3 hidden nodes, using our derived top 10, 20, 30, 40, 80 and 103 dimensions (genes). While the data set is small, each configuration achieved a 0% missclassification rate for cancer *vs.* noncancer. We then tested the neural network against an independent data set of nine frozen breast tumors from the University of Edinburgh, Scotland. **Using genes selected from within either the top 30 or 103 dimensions (top 30 or 103 discriminant genes), the neural network accurately predicted (before histopathology was available) that all nine samples were from histopathologically confirmed breast cancer (misclassification rate = 0%), not noncancer breast tissues.** We will use this approach to build similar predictive neural networks based on the cell culture data to identify antiestrogen sensitive and resistant gene expression patterns in known and unknown samples..

We have completed development and testing of an algorithm for data normalization. Normalization can adjust for the varying specific activities of the probe that result from small differences in the amount of RNA used to produce the probes, specific activity/labeling of the probes, batch-to-batch variation in chip production, and other interexperimental variations. While the most effective method is unclear, linear regression through the origin is widely accepted as the most theoretically valid (7). Other approaches include normalizing to the average expression level of either preselected "housekeeping" genes (based on biology), or of all signals on the array. As part of our suite of algorithms, we also developed a simple data normalization approach based on our implementation of regression through the origin (8):

$$y_i = ax_i + b \qquad \textbf{(Eq. 1)}$$

where, the data points in the floating data set are $\{x_1, x_2, x_3 \ldots, x_i\}$, and those in the reference set are $\{y_1, y_2, y_3 \ldots, y_i\}$.

Our method, which applies an approach similar to the "boosting" principle, differs from that of Chen *et al.* (7) in two ways. First, rather than forcing b=0, we iteratively estimate both *a* and *b*. Secondly, we select the genes for normalization iteratively, rather then predefining non-differentially expressed genes. We apply a bootstrap approach, starting with all genes, which alternates between estimating normalization coefficients and identifying an interim control gene subset for normalization. A factoring-shifting approach is used to estimate regression coefficients at each iteration, based on an interim control gene subset defined by a window function. The window function, which decays with alternate iterations, rejects outliers and measures the consistency of the matched neighborhoods, *i.e.,* the corresponding data for each gene in the reference and floating gene sets. By setting the window over the center of the scatter plot, we avoid using genes with very high or very low levels of expression in guiding normalization. Convergence is achieved when $b=0$ and $a=1$. For those cases where $b \approx 0$, our algorithm will arrive at a solution similar to that of Chen *et al.* Where $b \neq 0$, we believe that our algorithm will provide a more robust normalization.

Using our data, we compared our approach with that of Chen *et al.* and with normalization against the average of the signals on the blot. The mean squared error (MSE) of the regression coefficients were used as estimates of the goodness-of-fit of the regression to the data points. Both our method and that of Chen *et al.* produced similarly good fits (MSE = 3905, Chen *et al.*; MSE = 3892, our method), although, the precise location of the floating data points relative to the reference data may not be identical at convergence. Normalization against the average level of expression provided the least satisfactory regression coefficient (MSE = 8549). A manuscript describing this method has been accepted for publication in a informatics (not biomedical) journal.

TASK 3: *Begin to assess the likely functional relevance of representative members of these clusters and study their expression in human breast cancer biopsies.*

A. Obtain/generate reagents for the 1-2 candidates from the pilot study
B. Initiate pilot studies using transient transfection analyses
C. Initiate functional (transient) studies of candidates (5-10) from baseline study
D. Initiate functional (stable transfection) studies of candidates (1-5) from baseline study
E. Initiate functional (transient) studies of candidates (1-5) from treatment study
F. Initiate functional (stable transfection) studies of candidates (1-5) from overall analysis (only if new candidates are identified)

We continue to investigate the functional relevance of those genes/proteins that receive sufficient priority. This includes transient transfection studies with promoter-reporter constructs (for transcription modulating factors) and stable transfections to assess functional relevance. We have continued to work on the candidate genes involved in estrogen and antiestrogen resistance as described in the original application. These include nucleophosmin (*NPM*), interferon regulatory factor-1 (IRF-1), cAMP response element binding activities (CRE; induced by the human X-box binding protein-1 - hXBP-1) and nuclear factor kappa B (NFκB). We have completed studies

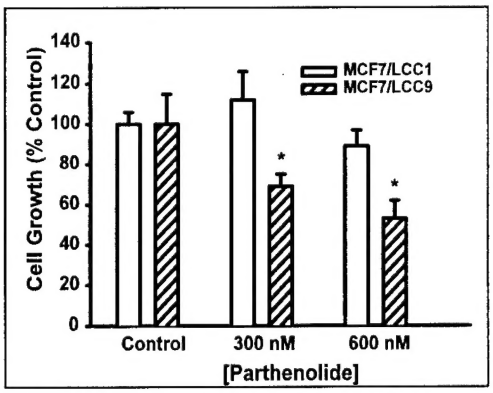

Fig 1: *Parthenolide inhibits proliferation of MCF7/LCC9 but not MCF7/LCC1 cells. *p<0.01 vs. control, n=4.*

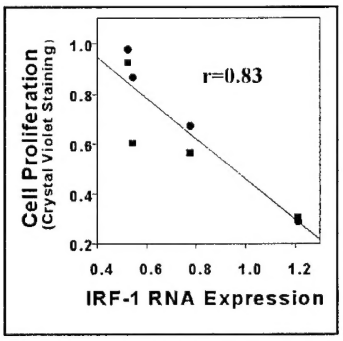

Fig 2: *IRF-1 expression is inversely correlated with the rate of cell proliferation. Squares = data from expt 1; circles=data from expt 2.*

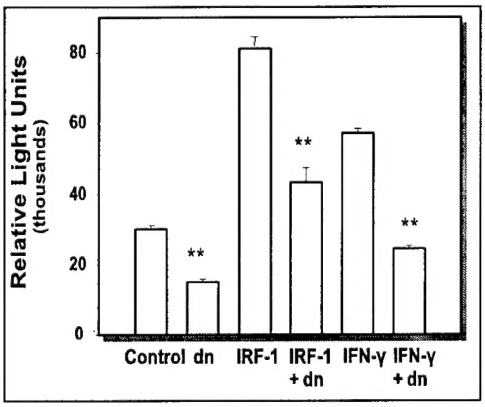

Fig 4: *Activity of the IRF-dominant negative (dn). **p<0.001 for dn vs. ctr and effects of dn on IRF-1 & IFN-γ (n=4).*

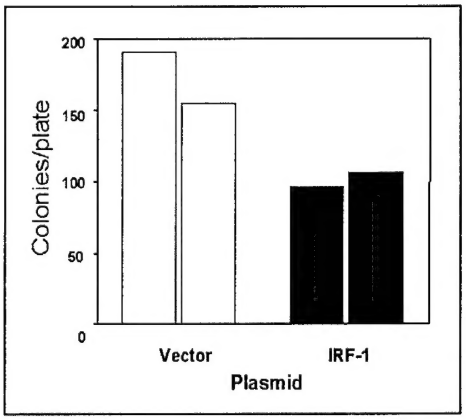

Fig 3: *Reduced anchorage-dependent colony formation in pooled IRF-1 transfectants compared with G418-resistant controls. data from two independent experiments.*

showing that antiestrogen resistant MCF7/LCC9 cells, which overexpress NFκB transactivation (promoter-reporter activity), are more sensitive to the growth inhibitory effects of Parthenolide, a specific inhibitor of NFκB (Fig 1). Growth inhibition was assessed using a dye-based assay that effectively estimates cell number. These data are consistent with our hypothesis that increased NFκB activation in these cells contributes to their ability to survive prolonged antiestrogen exposure.

Initially, we have chosen to focus primarily on studies of IRF-1. We have completed our studies of the IRF-1 transfectants. The full length IRF-1 cDNA was cloned into an expression vector placing its expression under the direction of the constitutive CMV promoter. IRF-1 was overexpressed using standard transfection methods (9). Overexpression of IRF-1 inhibits the rate of cell proliferation (Fig 2) and anchorage-dependent colony formation in antiestrogen and estrogen responsive MCF-7 cells (Fig 3). In Fig 2, we estimated the cell population doubling time and plotted these as a function of IRF-1 mRNA expression. A correlation coefficient was estimated, showing that the higher the level of IRF1 expression, the slower the cells grow ($r=0.83$). In Fig 3, we measured anchorage-dependent growth and counted the number of discrete colonies. IRF-1 transfectants produced fewer colonies than controls. These data are consistent with IRF-1 acting as a tumor suppressor gene in breast cancer, and are consistent with our initial hypotheses.

We have designed, built and tested a dominant negative IRF-1 construct (dnIRF-1) comprising the IRF-1 cDNA without the transactivation domain. Fig 4 shows preliminary data that dnIRF-1 is a potent inhibitor of endogenous and interferon-induced IRF-1 transcriptional activities (promoter reporter assay). These were done using standard transient transfection methods. We also stably overexpressed dnIRF-1 using the same vector and methods for overexpressing IRF-1. These cells have been injected into NCr athymic nude mice, and we are currently assessing the ability of dnIRF-1 overexpression to increase the tumorigenicity of MCF-7 cells. Initial studies suggest that these transfected cells grow more quickly *in vitro* and may be more tumorigenic *in vivo*. We hope to complete these studies and submit a manuscript on dnIRF-1 within the next 12 months. The mature data will be included in our next annual report.

Key Research Accomplishments (bulleted)

- Submitted manuscript describing data from gene microarray and SAGE studies based on the data presented in the previous report. These data show the altered regulation of X-box binding protein-1, NFκB, NPM and IRF-1 in acquired antiestrogen resistance (manuscript submitted).

- Collected RNA and obtained microarray data on the first series of RNA populations from resistant and parental cell cultures.

- Completed studies implicating IRF-1 as a tumor suppressor gene potentially involved in acquired antiestrogen resistance.

- Completed development of a new algorithm based on regression through the origin for normalizing gene expression microarray data (manuscript in press).

- Published a major review on cellular and molecular mechanisms of antiestrogen resistance.

- Completed a pilot study showing our ability to generate accurate predictive neural networks based on gene expression microarray data. The neural network predictors that can accurately identify the phenotype of unknown samples as being cancer or noncancer.

Reportable Outcomes

Reportable outcomes are presented as manuscripts and abstracts.

Manuscripts and Abstracts

We have published several studies directly related to the funded work. In addition to studies directly funded, we also evaluated the effects of Tamoxifen on the development of the normal rodent mammary gland (publication #5 below). Since such exposures may affect subsequent sensitivity to tumorigenesis and the responsiveness of any tumors (chemically induced or spontaneous) to antiestrogens, we include that publication in the listing below. Tissues from some of these rodents may be available for arraying in future years.

Manuscripts

1. Clarke, R., Leonessa, F., Welch, J.N., & Skaar, T.C. "Cellular and molecular pharmacology of antiestrogen action and resistance." *Pharmacol Rev*, 53: 25-72, 2001.

2. Clarke, R. & Dickson, R.B. "Animal models of endocrine responsive and unresponsive breast cancers". In: *"Endocrine Management of Breast Cancer"*, Eds: Robertson, J. & Hayes, D.F., Isis Medical Media Ltd., Faringdon, U.K., in press.

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4. Gu, Z., Lee, R.Y., Skaar, T.C., Bouker, K.B., Welch, J.N., Lu, J., Liu, A., Davis, N., Leonessa, F., Brünner, N., Wang, Y. & Clarke, R. "Molecular profiles of antiestrogen resistance implicate NFκB, cAMP response element binding, nucleophosmin and interferon regulatory factor-1." submitted.

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Abstracts

6. Davis, N., Gu, Z., Hanfelt, J., Hurley, C., Xiao, H., Gray, F., Flessate-Harley, D., & Clarke, R. "Gene expression profiles associated with antiestrogen responsive versus resistant breast cancer cells." *Proc Am Assoc Cancer Res*, 41: 159, 2000.

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Conclusions

We have made good progress in our studies on the molecular characterization of antiestrogen resistance. Progress is evident in our productivity as measured by publications, development of a new normalization algorithm for microarray analysis, and additional preliminary data. We expect to have completed the entire analysis (data collection and analysis) of the untreated populations within the next few months. Concurrently, we will begin collecting RNA from the treated cells. The study is on-track and the amount of data accumulating and being analyzed is considerable. Several new algorithms underdevelopment are showing good performance in our very preliminary analyses of published high dimensional data sets. We hope to report on some of these next year in more detail. Our data with NFκB, IRF-1 and the dnIRF-1 are encouraging and suggest we may be on the right track to identifying new signal transduction pathways associated with acquired antiestrogen resistance. For example, these data show that resistant cells are more sensitive to inhibition of NFκB. Overexpression of IRF-1, which is suppressed by estrogens and induced by antiestrogens, is associated with reduced cell proliferation. The dnIRF-1 provide an opportunity to further explore some of the mechanistic effects of this gene in acquired antiestrogen resistance.

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